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Anti-Platelet Pentacyclic Triterpenoids from Leaves of *Campsis grandiflora*

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Five pentacyclic triterpenoids, oleanolic acid (**1**), hederagenin (**2**), ursolic acid (**3**), tormentic acid (**4**) and myrianthic acid (**5**), were isolated from the methanol extract of the leaves of *Campsis grandiflora*, and structures of the compounds were established by the spectroscopic methods. Compounds **2**, **3**, **4**, and **5** were isolated for the first time from the genus *Campsis*. All of the compounds (IC_{50}: 45.3, 32.8, 82.6, 42.9 and 46.2 μM respectively) were as equivalently inhibitive as acetylsalicylic acid (IC_{50}: 57.0 μM) on epinephrine induced platelet aggregation.

Key words: *Campsis grandiflora*, Pentacyclic triterpenoids, Anti-platelet

INTRODUCTION

Blood platelet plays important roles not only in normal hemostatic process (Rand *et al.*, 2003), but also in thrombosis formation, which is the pivotal contributor to pathogenesis of many circulatory diseases (Ni *et al.*, 2003). Thus anti-platelet compounds have wide therapeutic potential for various circulatory diseases.

Campsis grandiflora (Thunb.) K. Schum (Bignoniaceae), which is also known as its synonym, *Campsis chinensis* (Lam.) Voss is creeping plant with large, deep orange to red flowers. The flowers of this plant have long been used as herbal remedies in traditional Chinese Medicine for treatment of diseases caused by blood stagnation (Dictionary of Chinese Crude Drugs, 1997). The isolateion of iridoids and phenyl propanoid glycosides from the leaves (Imakura *et al.*, 1984, 1985a and 1985b; Guiso, 1982) and oleanolic acid (Zhao *et al.*, 2002) from the flowers of this plant were reported. In the continuous search for anti-platelet compounds from natural sources, five pentacyclic triterpenoids have been isolated from the MeOH extract of this plant.

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MATERIALS AND METHODS

Instruments and reagents

Melting point was determined on a BÜchi B-540 melting point apparatus and uncorrected. IR spectra were recorded on a Jasco FT/IR-5300 spectrometer on KBr. Mass spectra were taken with a Hewlett Packard model 5989 B GC/MS system. ^{1}H- and ^{13}C-NMR spectra were taken at 300 MHz and 75.5 MHz respectively with a Varian Gemini-2000 spectrometer using TMS as an internal standard. $[\alpha]_D$ was measured on a Jacoso P-1020 autopolarimeter. Platelet count was determined on an Excell 18 Hematology Analyzer (MWI, Inc., DANAM Electronics, USA). Platelet aggregation was measured on a platelet aggregometer (Model 490-X, Chrono-Log Corp., Havertown, USA). Silica gel 60 (Art 7734, 70-230 mesh, and Art 7729, <230 mesh, Merck DGaA, Germany) was used for open chromatography. TLC was performed on silica gel 60 F_{254} (Merck DGaA, Germany). Collagen and ADP (adenosine 5'-diphosphate dicyclohexylammonium salts) were purchased from Chrono-Log Corp. (Havertown, USA). Epinephrine, sodium arachidonate (AA) and U46619 (9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$) were obtained from Sigma Chem. Corp. (St. Louis, USA).

Animals

The rats (Sprague-Dawley, 250±20 g) were fed with a diet of animal chow and tap water and were housed at 20±2°C and 55±5% humidity in a 12 h light-dark cycle in accordance with the Guide for the Care and Use of

Laboratory Animals by Seoul National University.

Plant materials

C. grandiflora were collected in early July (2002) in Seoul, and was identified by Prof. Emeritus Hyung Joon Chi, Natural Products Research Institute, Seoul National University. The voucher specimens (JLJ-020010) were deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Korea.

Extraction and isolation

The dried leaves of *C. grandiflora* (3.8 kg) were extracted with methanol three times for six hours each. The MeOH extract concentrated *in vacuo* (400 g) was partitioned with $CHCl_3$ and H_2O, and the $CHCl_3$ layer (120 g) after concentration was partitioned with hexane and 90% MeOH to obtain hexane fraction (40 g) and MeOH fraction (80 g). MeOH fraction was subjected to silica gel chromatography eluting with $CHCl_3$-MeOH (95:5-70:30), affording compound **1** (30 mg), **2** (15 mg), **3** (50 mg), **4** (10 mg), and **5** (15 mg).

Hederagenin (2)

White powder, mp >300°C; $[\alpha]_D^{23}$ + 76.7° (*c* 0.14, MeOH); IR ν_{max} cm^{-1} (KBr): 3455 (OH), 2944 (CH_3), 1699 (CO), 1466, 1389; EI-MS (rel. int.) *m/z*: 472 $[M]^+$ (3.8), 454 $[M-H_2O]^+$ (1.0), 426 $[M-HCOOH]^+$ (1.0), 248 [D/E rings]$^+$ (93.8), 55 (100.0); ^{1}H-NMR (pyridine-d_5) δ: 5.48 (1 H, *t-like s*, H-12), 4.18 (1 H, *br t, J*=9.6 Hz, H-3α), 4.17 and 3.70 (1H each, *d, J*= 10.5 Hz, H-23), 3.29 (1H, *dd, J*= 3.6, 13.5 Hz, H-18), 1.22 (3H, *s*, H-27), 1.04 (3H, *s*, H-24), 1.03 (3H, *s*, H-26), 0.99 (3H, *s*, H-30), 0.96 (3H, *s*, H-25), 0.91 (3H, *s*, H-29); ^{13}C-NMR see Table I.

Ursolic acid (3)

White powder, mp 281~283°C; $[\alpha]_D^{23}$ + 72.9° (*c* 0.03, MeOH); IR ν_{max} cm^{-1} (KBr): 3430 (OH), 2924 (CH_3), 2855 (CH_3), 1694 (CO), 1458, 1387; EI-MS (rel. int.) m/z: 456 $[M]^+$ (2.4), 438 $[M-H_2O]^+$ (1.2), 410 $[M-HCOOH]^+$ (1.0), 248 [D/E rings]$^+$ (100); ^{1}H-NMR (pyridine-d_5) δ: 5.49 (1H, *t-like s*, H-12), 3.46 (1H, *dd, J*= 7.2, 8.4 Hz, H-3α), 2.63 (1H, *d, J*= 11.4 Hz, H-18), 1.24 (3H, *s*, H-23), 1.22 (3H, *s*, H-27), 1.05 (3H, *s*, H-26), 1.02 (3H, *s*, H-24), 0.99 (3H, *d, J* = 6.3 Hz, H-30), 0.95 (3H, *d, J* = 5.4 Hz, H-29), 0.88 (3H, *s*, H-25); ^{13}C-NMR see Table I.

Tormentic acid (4)

White powder, mp 272~273°C; $[\alpha]_D^{23}$ + 29.1° (c 0.13, MeOH); IR ν_{max} cm^{-1} (KBr): 3447 (OH), 2932 (CH_3), 1696 (CO), 1458, 1389; EI-MS (rel. int.) *m/z*: 488 $[M]^+$ (1.2), 470 $[M-H_2O]^+$ (1.0), 442 $[M-HCOOH]^+$ (14.5), 424 $[M-HCOOH-H_2O]^+$ (1.2), 370 (11.9), 264 (4.0), 248 (92.9), 146 (100.0); ^{1}H-NMR (pyridine-d_5) δ: 5.58 (1H, *t-like s*, H-12), 4.09 (1H, *dt, J*=3.6, 9.3 Hz, H-2b), 3.37 (1H, *d, J*=9.3 Hz, H-3a), 3.12 (1H, *dt, J*=4.8, 12.6 Hz, H-16a), 3.04 (1H, *s*, H-18), 1.70 (3H, *s*, H-27), 1.42 (3H, *s*, H-29), 1.26 (3H, *s*, H-23), 1.11 (3H, *d, J*=5.7 Hz, H-30), 1.10 (3H, *s*, H-26), 1.07 (3H, *s*, H-24), 1.00 (3H, *s*, H-25); ^{13}C-NMR see Table I.

Table I. ^{13}C-NMR data of compounds **2~5**

Carbon No.	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**5**[b]
1	38.79	38.99	47.89	42.33	41.38
2	27.67	27.95	68.59	66.24	64.90
3	73.44	78.09	83.85	78.88	76.06
4	42.87	39.30[d]	39.84	42.17	43.51
5	48.63	55.73	55.95	43.54	46.79
6	18.59	18.69	18.99	18.43	17.74
7	32.97	33.48	33.51	33.19	32.51
8	39.76	39.40[d]	40.42	40.48	39.55
9	48.15	47.98	47.82	47.72	47.05
10	37.23	37.36	38.49	38.39	37.85
11	23.77[c]	23.54	24.11	24.09	23.37
12	122.58	125.56	127.93	127.91	126.94
13	144.82	139.15	139.95	139.97	138.84
14	42.18	39.87	42.14	41.88	41.33
15	28.34	28.59	29.26[f]	29.19	28.19
16	23.69[c]	24.81	26.37	26.33	25.35
17	46.65	42.40	48.28	48.24	47.05
18	42.99	53.46	54.59	54.56	53.36
19	46.45	39.30[d]	72.68	72.64	71.81
20	30.94	39.30[d]	42.36	42.65	41.57
21	34.22	30.97	26.93	26.90	26.12
22	33.24	37.18	38.49	38.38	37.47
23	67.96	28.72	29.33[f]	71.20	69.34
24	13.11	16.49	17.65	16.76[h]	16.65
25	15.96	15.58	16.87[g]	17.69[i]	16.85
26	17.48	17.43[e]	17.24	17.29[i]	17.05
27	26.16	23.83	24.68	24.66	24.27
28	180.16	179.91	180.65	180.67	179.13
29	33.24	21.33	27.09	27.04	26.59
30	23.84[c]	17.38[e]	16.77[g]	16.99[h]	16.48

a : in pyridine-d_5
b : in DMSO-d_6
c, d, e, f, g, h, i: values with the same symbols are inter-changeable.

Myrianthic acid (5)

White powder, mp >300°C; $[\alpha]_D^{23}$ + 33.9° (*c* 0.05, MeOH); IR ν_{max} cm^{-1} (KBr): 3451 (OH), 2934 (CH_3), 1696 (CO), 1458, 1389; EI-MS (rel. int.) *m/z*: 504 $[M]^+$ (1.1), 458 $[M-HCOOH]^+$ (3.3), 442 (1.2), 386 (3.6), 264 (3.6), 248 (92.9), 55 (100.0); ^{1}H-NMR (pyridine-d_5) δ: 5.57 (1H, *t-like s*, H-12). 4.26 (1H, *m*, H-2b), 4.12 (1H, *d, J*=2.4 Hz, H-3 b),

3.90, 3.73 (1H each, *d*, *J*=10.5 Hz, H-23), 3.09 (1H, *dt*, *J*=4.8, 13.2 Hz, H-16a), 3.02 (1H, *s*, H-18), 1.08 (3H, *d*, *J*=2.4 Hz, H-30), 1.65 (3H, *s*, H-27), 1.39 (3H, *s*, H-29), 1.11, 1.00, 0.84 (3H each, *s*, H-24, 25, 26); ^{1}H-NMR (DMSO-d_6) δ: 5.17 (1H, *t-like s*, H-12), 3.70 (1H, *m*, H-2b), 3.41 (1H, *br s*, H-3b), 2.36 (1H, *s*, H-18), 1.28 (3H, *s*, H-27), 1.07 (3H, *s*, H-29), 0.85 (3H, *d*, *J*=6.3 Hz, H-30), 0.90, 0.70, 0.68 (3H each, *s*, H-24, 25, 26); ^{13}C-NMR see Table I.

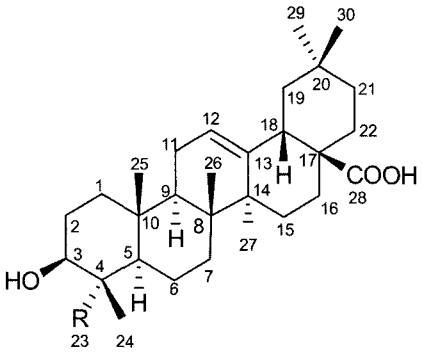
Anti-platelet aggregating activity

Rat blood was drawn from heart after surgery using syringes containing 0.1 part of 2.2% sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200×g for 10 min. PRP was diluted with saline to adjust the final platelet number to 400-450× 10^6/mL. The degree of aggregation was measured with Platelet Aggregometer. After 3 min preincubation of the adjusted PRP, sample (dissolved in DMSO) or vehicle was added and an aggregation inducing agent ADP (3-4 μM), collagen (2-3 μg/mL) or epinephrine (3-4 μM) was added at 30 sec after the sample addition. Epinephrine induced aggregations were measured by the method previously prescribed (Yun-Choi *et al.*, 2000). Briefly, sample was added 30 sec before the addition of the threshold concentration of collagen (0.8-1.0 μg/mL) and epinephrine (3-4 μM) was added 30 sec after the addition of collagen. The reduction in turbidity of PRP was observed as the aggregation processed. AA (20-40 μM) or U46619 (3-4 μM) induced aggregations were also measured in the presence of the threshold concentration of collagen. The minimum inducer concentration that elicited maximal aggregation was employed as the control for each PRP. The concentration at which each compound causing 50% inhibition (IC_{50}) was determined from the Regression Wizard from the SigmaPlot equation library.

RESULTS AND DISCUSSION

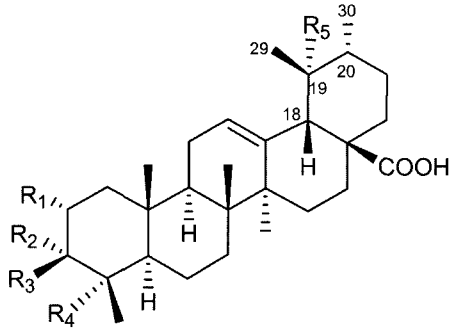
The MeOH extract of leaves of *C. grandiflora* was fractionated to three solvent fractions, hexane, 90% MeOH and H_2O soluble fraction. Of the three fractions, the 90% MeOH fraction, which showed inhibitory effects on platelet aggregation, provided five pentacyclic triterpenoids. The structure of each compound was identified with the aid of IR, EIMS, 1D and 2D NMR spectroscopic methods. Compound **1** was identified as oleanolic acid (Han *et al.*, 1987; Kizu and Tomimori, 1982), the isolation of which was previously reported from the flower of the present plant (Zhao *et al.*, 2002).

Compound **2** was obtained as white powder. The IR spectrum showed presence of hydroxyl group at 3455 cm^{-1} and acid carbonyl group at 1699 cm^{-1}. The EI-MS



1 R=CH_3

2 R=CH_2OH



3 R_1=H; R_2=H; R_3=OH; R_4= CH_3; R_5=H

4 R_1=OH; R_2=H; R_3= OH; R_4= CH_3; R_5=OH

5 R_1=OH; R_2=OH; R_3= H; R_4= CH_2OH; R_5=OH

Fig. 1. Compounds isolated from *C. grandiflora*

spectrum showed a molecular ion peak at *m/z* 472, and other significant fragment ion peaks at 454 $[M-H_2O]^+$, 426 $[M-HCOOH]^+$ and 248 [D/E rings]$^+$. The ^{1}H-NMR spectrum of this compound showed six tertiary methyl groups at δ 0.91~δ 1.22, a double doublet at δ 3.29 (*J* = 3.6 and 13.5 Hz, H-18), and a triplet-like singlet of olefinic proton at δ 5.48 (H-12), suggested the presence of the olean-12-en type structure. Two doublets protons at δ 4.17 and δ 3.70 with *J* = 10.5 Hz were assignable to two hydroxylated vicinal protons, and the broad triplet at δ 4.18 (1H, *J* = 9.6 Hz) was suggestive of the presence of 3β-hydroxyl group. The ^{13}C-NMR spectrum showed two olefinic signals at δ 144.82 (C-13) and δ 122.58 (C-12), one acid signal at δ 180.16 (C-28), two oxygenated carbons at δ 73.44 (C-3) and δ 67.96 (C-23), and signals at δ 46.65, δ 46.45 and δ 42.99. Thus, **2** was identified as hederagenin, the (3β, 4α)-3, 23-dihydroxy-olean-12-en-28-oic acid (Jung *et al.*, 1993; Kizu and Tomimori, 1982).

Compound **3** was obtained as white powder. The IR spectrum showed presence of hydroxyl group at 3430 cm^{-1}, methyl group at 2924 cm^{-1} and acid carbonyl group at 1694 cm^{-1}. The EI-MS spectrum showed a molecular ion peak at *m/z* 456, and other significant fragment ion

peaks at 438 $[M-H_2O]^+$, 410 $[M-HCOOH]^+$ and 248 [D/E rings]$^+$. The ^{1}H-NMR spectrum of **3** showed five tertiary methyl singlets at δ 0.88~δ 1.24, two secondary methyl doublets at δ 0.95 (J = 5.4 Hz) and δ 0.99 (J = 6.3 Hz). A doublet proton at δ 2.63 (H-18) and an olefinic proton at δ 5.49 (H-12), which strongly suggested the presences of the urs-12-en type structure. A double doublet (J = 7.2 and 8.4 Hz) proton signal at δ 3.46 suggested the 3β-hydroxyl functionality. The ^{13}C-NMR spectrum of **3** showed one acid carbon signal at δ 179.91 (C-28), two olefinic signals at δ 139.15 (C-13), δ 125.56 (C-12), and an oxygenated carbon at δ 78.09 (C-3) together with peaks at δ 42.40 (C-17) and δ 53.46 (C-18), which strongly supports the structure of urs-12-en-17-carboxylic acid. Accordingly, **3** was identified as ursolic acid, (3β-hydroxy-urs-12-en-28-oic acid) by direct comparison of the spectral data with the literatures values (Choi *et al.*, 1991; Numata *et al.,* 1989).

Compound **4** was obtained as white powder. The IR spectrum was similar to that of **3**. The EI-MS spectrum showed a molecular ion peak at *m/z* 488. The ^{1}H-NMR spectrum showed six tertiary methyl singlets at δ 1.00~δ 1.70, one methyl doublet at δ 1.11 (J = 5.7 Hz), and an olefinic proton at δ 5.58. One singlelet proton signal at δ 3.04, and another proton at δ 3.12 (*dt, J*= 6.1, 13.5 Hz) assignable to 16α proton due to anisotropic effect of 19α-OH were also observed, which suggested to retain an 19α-hydroxy-ursenoic acid framework (Aimi *et al.,* 1989; Liang *et al.*, 1989). Two oxygenated methine protons at δ 4.09 (*dt, J*=3.6, 9.3 Hz) and δ 3.37 (*d, J*=9.3 Hz) were suggestive of 2α and 3β dihydroxy structure. The ^{13}C-NMR spectrum again suggested the trihydroxylated ursenoic acid structure with the presence one carboxyl carbon signal at δ 180.65 (C-28), two olefinic carbon signals at δ 139.95 (C-13) and δ 127.93 (C-12), and three hydroxylated carbons at δ 68.59 (C-2), δ 83.85 (C-3) and δ 72.68 (C-19). The identity of **4** as tormentic acid (2α, 3β, 19α-trihydroxy-urs-12-en-28-oic acid) was confirmed by comparison of reported spectral data (Numata *et al.,* 1989; Kitajima and Tanaka, 1993; Taniguchi *et al.,* 2002).

Compound **5** was obtained as white powder. The IR spectrum was similar to that of **4**. The EI-MS spectrum showed a molecular ion peak at *m/z* 504. The ^{1}H-NMR (pyridine-d_5) spectrum showed five tertiary methyl groups at δ 0.84~δ 1.65, and one methyl doublet at δ 1.08 (J = 2.4 Hz). Furthermore, the presence of one singlelet proton signal at δ 3.02 (H-18), one olefinic proton at δ 5.57 (H-12) and an another proton at 3.09 (H-16α) demonstrated the presence of 19α-hydroxy-ursenoic acid structure as in compound **4**. Two methine protons with hydroxyl groups were exhibited the signals at δ 4.26 (*m*) and δ 4.12 (*d*, J = 2.4 Hz), which were assignable to 2α and 3α protons. The ^{13}C-NMR (pyridine-d_5) spectrum confirmed the presence of a pair of olefinic carbons at δ 127.91 (C-12) and δ 139.97 (C-13), one carboxylic acid carbon at δ 180.67 (C-28) and four hydroxylated carbons at δ 66.24 (C-2), δ 78.88 (C-3), δ 72.64 (C-19) and δ 71.20 (C-23) on the ursene structure. All the above mentioned data suggested myrianthic acid (2α, 3α, 19α, 23-tetrahydroxy-urs-12-en-28-oic acid) for the structure of compound **5**. ^{1}H- and ^{13}C-NMR spectra were also obtained in DMSO-d_6 and were compared with its reported spectral data (Wandji *et al.*, 2003) to confirm the structure.

This is the first report on the isolation of compounds **2**, **3**, **4**, and **5** from the genus *Campsis,* although the presence of ursolic acid was detected from *Flos campsis* by the method of micellar electrokinetic capillary chromatography (Liu *et al.*, 2003).

The inhibitory effects of the five compounds **1~5** on rat platelet aggregation were compared with those of acetyl salicylic acid (ASA), one of the well-known inhibitors of platelet aggregation (Table I). Most of the tested compounds showed mild but dose-dependent inhibitory activities on ADP, collagen, epinephrine, arachidonic acid (AA) and U46619, a synthetic PGH_2/TXA_2 receptor agonist, induced rat platelet aggregation. All of the five compounds **1~5** showed inhibitory effects on epinephrine induced platelet aggregation (IC_{50}: 45.3, 32.8, 82.6, 42.9 and 46.2 μM respectively), which were approximately equivalent to ASA (IC_{50}: 57.0 μM). But all of them were only very mildly inhibitory to ADP, collagen, AA and U46619 induced aggregation (IC_{50}: >500 μM). ASA is thought to show inhibitory effects on both epinephrine and AA induced aggregation by reducing the TXA_2 synthesis through acting on cycloxygenase. Since the present compounds **1~5** were much less active to AA induced aggregation than to epinephrine induced aggregation, they should present anti-platelet function with other mechanism than ASA. However, the precise mechanism of action of these compounds should further be clarified.

Table II. Platelet anti-aggregating activities of compounds isolated from *C. grandiflora*

Compounds	IC_{50} (μM)				
	ADP[a]	Collagen[b]	Epinephrine[c,f]	AA[d,f]	U46619[e,f]
ASA[g]	>1000	460	57.0	63.0	350
1	>1000	>1000	45.3	>1000	>1000
2	674	>1000	32.8	>1000	721
3	>1000	511	82.6	669	>1000
4	>1000	>1000	42.9	>1000	>1000
5	>1000	>1000	46.2	>1000	>1000

[a]ADP 3-4 μM, [b]collagen 2-3 μg/mL, [c]epinephrine 3-4 μM, [d]sodium arachidonate 20-40 μM, [e]U46619 3-4 μM, [f]with the threshold concentration of collagen (collagen 0.8-1.0 μg/mL), [g]ASA; acetylsalicylic acid.

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